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An engineered promoter driving expression of a microbial avirulence gene confers recognition of TAL effectors and reduces growth of diverse *Xanthomonas* strains in citrus

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SUMMARY

Xanthomonas citri ssp. *citri* (*X. citri*), causal agent of citrus canker, uses transcription activator-like effectors (TALEs) as major pathogenicity factors. TALEs, which are delivered into plant cells through the type III secretion system (T3SS), interact with effector binding elements (*EBE*s) in host genomes to activate the expression of downstream susceptibility genes to promote disease. Predictably, TALEs bind *EBE*s in host promoters via known combinations of TALE amino acids to DNA bases, known as the TALE code. We introduced 14 *EBE*s, matching distinct *X. citri* TALEs, into the promoter of the pepper *Bs3* gene ($ProBs3_{1EBE}$), and fused this engineered promoter with multiple *EBE*s ($ProBs3_{14EBE}$) to either the *β-glucuronidase* (*GUS*) reporter gene or the coding sequence (cds) of the pepper gene, *Bs3*. TALE-induced expression of the *Bs3* cds in citrus leaves resulted in no visible hypersensitive response (HR). Therefore, we utilized a different approach in which $ProBs3_{1EBE}$ and $ProBs3_{14EBE}$ were fused to the *Xanthomonas* gene, *avrGf1*, which encodes a bacterial effector that elicits an HR in grapefruit and sweet orange. We demonstrated, in transient assays, that activation of $ProBs3_{14EBE}$ by *X. citri* TALEs is T3SS dependent, and that the expression of AvrGf1 triggers HR and correlates with reduced bacterial growth. We further demonstrated that all tested virulent *X. citri* strains from diverse geographical locations activate $ProBs3_{14EBE}$. TALEs are essential for the virulence of *X. citri* strains and, because the engineered promoter traps are activated by multiple TALEs, this concept has the potential to confer broad-spectrum, durable resistance to citrus canker in stably transformed plants.

Keywords: *avrGf1*, effector binding element (*EBE*), hypersensitive response (HR), pathogen-inducible promoter, transcription activator-like effector (TALE).

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INTRODUCTION

Bacterial plant pathogens make use of a transmembrane needle-like structure, the type III secretion system (T3SS), to inject an assortment of effector proteins into host mesophyll cells (Chaudhari *et al.*, 2014; Hogenhout *et al.*, 2009). In susceptible plants, these effector proteins target host functions in order to suppress defence or to generate a favourable environment that promotes bacterial colonization (Zhou and Chai, 2008). Some plant genotypes contain resistance (*R*) genes that mediate the recognition of the activity or structure of bacterial effectors and orchestrate a plant defence response that typically culminates in a localized cell death reaction, known as the hypersensitive response (HR) (Dickman and Fluhr, 2013).

One particular effector class which is prominent in *Xanthomonas* species is the transcription activator-like effectors (TALEs). It is exemplified by the well-studied TALE AvrBs3 from *Xanthomonas euvesicatoria* (*Xeu*), the causal agent of bacterial leaf spot in pepper (Stall *et al.*, 2009). Following injection into the plant cell, TALEs localize to the nucleus, bind to corresponding effector binding elements (*EBE*s; Antony *et al.*, 2010) present in the host genome, and activate the transcription of downstream host susceptibility (*S*) genes to promote disease (Boch and Bonas, 2010; Boch *et al.*, 2014; Bogdanove *et al.*, 2010; Doyle *et al.*, 2013; Mak *et al.*, 2013; Schornack *et al.*, 2013). TALEs differ predominantly in their central DNA-binding domain, which is composed of near-identical 33–35-amino-acid repeats arranged in tandem. Each TALE repeat binds to a single DNA base, and repeat residue 13, the base-specifying residue (BSR), determines the base preference of a given repeat (de Lange *et al.*, 2014). The set of correlations between BSRs and their cognate base(s) is known as the TALE code, and facilitates the prediction of *EBE*s based solely on the BSR composition of a given TALE (Boch *et al.*, 2009; Moscou and Bogdanove, 2009).

Analysis of the TALE protein AvrBs3 in the context of the pepper (*Capsicum annuum*) cultivar Early California Wonder (ECW)

uncovered putative pepper *S* genes that are believed to promote bacterial spot disease (Kay and Bonas, 2009; Kay *et al.*, 2007; Marois *et al.*, 2002). However, in the pepper cultivar ECW-30R, which is an isogenic variant of ECW, AvrBs3 triggers an immune reaction. Recognition of AvrBs3 in ECW-30R is mediated by the bacterial spot *R* gene *Bs3*. *Bs3* contains an *EBE* compatible to AvrBs3 which, on binding, transcriptionally activates the *Bs3* promoter. AvrBs3-mediated activation of *Bs3* triggers the HR and inhibits the growth and spread of *Xeu* (Jordan *et al.*, 2006; Römer *et al.*, 2007).

Pepper *Bs3* is one of five known plant *R* genes that are transcriptionally activated by TALEs (Zhang *et al.*, 2015). Such *R* genes are modular systems consisting of a pathogen-inducible promoter driving a gene that encodes an executor R protein (Boch *et al.*, 2014). As indicated by their designation as executor R proteins, they are functionally distinct from constitutively expressed R proteins, as executor R proteins are not involved in effector recognition, but only in the execution of the plant immune reaction. The five cloned *R* genes encoding executor proteins have been isolated from pepper and rice, and mediate resistance to xanthomonads infecting these plant species (Gu *et al.*, 2005; Römer *et al.*, 2007; Strauß *et al.*, 2012; Tian *et al.*, 2014; Wang *et al.*, 2015). Because of the dearth of mechanistic information on how executor R proteins induce resistance, it is not possible to predict whether or not executor R proteins remain functional on transfer to other plant species. Similarly, the microbial pathogens against which they possibly confer resistance cannot be predicted.

We studied Asiatic citrus canker, one of the most economically damaging diseases of the citrus industry and for which no effective means of genetic or chemical control exists. Citrus canker is caused by the bacterial pathogens *Xanthomonas citri* ssp. *citri* (hereafter referred to as *X. citri* or *Xcc*), which are designated as A strains, and *X. fuscans* pv. *aurantifolii*, which are designated as B and C strains. Citrus canker is difficult to control, and the limited genetic resources of plant resistance that have been identified have not been introgressed into commercial varieties (Khalaf *et al.*, 2008; Machado *et al.*, 2011; Rybak *et al.*, 2009). Based on our previous results demonstrating that the *Bs3* promoter can be engineered to contain several functional *EBE*s corresponding to and inducible by specific TALEs (Römer *et al.*, 2009a), and our ability to deduce *EBE*s for any TALE by the TALE code (Boch *et al.*, 2009; Hu *et al.*, 2014; Li *et al.*, 2014), we investigated whether this knowledge could be used to engineer durable and broad-spectrum resistance to *X. citri*.

Because all citrus canker strains depend on TALEs for complete virulence, in particular PthA4 and its close homologues (Da Silva *et al.*, 2002; Hu *et al.*, 2014), we hypothesized that a pepper *Bs3* promoter derivative that contains multiple *EBE*s matching distinct *X. citri* TALEs could mediate the perception of the corresponding *X. citri* strains and could induce an HR when driving the expression of the *Bs3* coding sequence (cds). Yet, we found that *Agrobacterium*-mediated delivery of a *35S* promoter-driven *Bs3* cds did not cause a visible HR in citrus. The functionality of other known pepper and rice executor R proteins in citrus is unknown. However, previous studies have shown that constitutive *in planta* expression of the *X. citri* effector gene *avrGf1* triggers HR in citrus (Figueiredo *et al.*, 2011). In the present study, we have tested whether *avrGf1* can be used as an executor module in an engineered, TALE-inducible promoter trap as a strategy to confer resistance to citrus canker. Indeed, we demonstrated that our engineered promoter mediates the recognition of multiple distinct *X. citri* TALEs from a world-wide collection of citrus canker strains, and that TALE-triggered expression of this AvrGf1 results not only in a visible HR, but also in reduced *X. citri* growth in transient assays. These data support a genetic control strategy for this major citrus disease and also demonstrate a novel modular concept to engineer promoter traps in plant species for which suitable executor R proteins are not available.

RESULTS

TALE-dependent activation of the pepper *Bs3* promoter in citrus

Previously, we have shown that the TALE protein AvrBs3 from *Xeu* binds to a compatible *EBE* (EBE_{AvrBs3}) present in the pepper *Bs3* promoter [herein defined as $ProBs3_{1EBE}$ (subscript indicates the AvrBs3-compatible *EBE* in the *Bs3* promoter)], resulting in the transcriptional activation of *Bs3* (Römer *et al.*, 2009a). Given that the *Bs3* promoter is transcriptionally inactive in the absence of a matching TALE (Römer *et al.*, 2007, 2009a,b), this promoter is particularly well suited to study TALE-dependent transcriptional activation *in planta*. We fused $ProBs3_{1EBE}$ upstream of a *β-glucuronidase* (*GUS*) reporter gene to examine TALE-dependent promoter activation in citrus (Fig. S1, see Supporting Information). For these studies, we used a *GUS* reporter gene variant containing an intron, herein designated as *GUSi*, which excludes the expression of a functional GUS protein in *Agrobacterium tumefaciens*. Use of the *GUSi* reporter gene ensures that any measured GUS activity is caused by the *in planta* expression of a processed *GUSi* transcript. To study TALE-dependent promoter activation, young grapefruit leaves were infiltrated first with a bacterial suspension of *A. tumefaciens* containing the promoter-reporter construct $ProBs3_{1EBE}$*:GUSi*. Five hours later, the inoculated leaf sections were infiltrated with a set of *Xcc*306 strains, each delivering a different set of TALEs [*Xcc*306 (PthA1, 2, 3 and 4), *Xcc*306Δ*pthA1–4* (no TALEs), *Xcc*306Δ*pthA1–4:avrBs3* (AvrBs3 only) and *Xcc*306*:avrBs3* (AvrBs3, PthA1, 2, 3 and 4)]. Five days after inoculation, the infiltrated leaf sections were assayed for GUS activity

(Fig. 1, left side). GUS activity was highest in leaves subsequently inoculated with *Xcc*306Δ*pthA1–4:avrBs3*, which expresses AvrBs3. Given that AvrBs3 and the corresponding EBE_{AvrBs3} are present in the subsequently inoculated *Xanthomonas* strain and promoter-reporter construct, respectively, the observed high GUS activity matches the expectations. In contrast, GUS activity by *Xcc*306 carrying *avrBs3* was extremely low, indicating an antagonistic effect of PthA proteins on the activation of EBE_{AvrBs3} by AvrBs3. The isogenic strain *Xcc*306Δ*pthA1–4*, which lacks AvrBs3, did not activate the GUS reporter to measurable levels, demonstrating that activation of the *Bs3* promoter in citrus by *Xcc*306Δ*pthA1–4:avrBs3* is dependent on AvrBs3. Interestingly, low promoter activation was also observed when $ProBs3_{1EBE}$*:GUSi* was subsequently inoculated with *Xcc*306 which contains PthA1–4 (Fig. 1, left side), suggesting that not only AvrBs3, but also at least one of the *X. citri*-delivered *PthA* TALE proteins, is capable of activating the *Bs3* promoter to a low, but measurable, level.

Xanthomonas citri TALEs strongly activate a promoter containing corresponding tandem-arranged *EBE*s

Previously, we have shown in transient expression systems that the *Bs3* promoter can be engineered to accommodate multiple *EBE*s, each autonomously binding corresponding TALEs and mediating the transcription of a downstream coding sequence (Römer *et al.*, 2009a). In an attempt to generate a promoter that would mediate the recognition of a broad range of *X. citri* strains, the *Bs3* promoter was engineered to accommodate 14 different *EBE*s (Fig. S2, see Supporting Information). These included the four *EBE*s corresponding to PthA1–4, which are present in the Brazilian *Xanthomonas* strain *Xcc*306 used in these assays. The *EBE*s were designed according to the TALE code (Boch *et al.*, 2009; Moscou and Bogdanove, 2009) and correspond to *X. citri* TALEs deposited in protein databases or determined in the present study (Table S1, see Supporting Information). This complex promoter with 14 *EBE*s, herein designated as $ProBs3_{14EBE}$, was fused upstream of the *GUSi* reporter gene, and TALE-dependent promoter activation was studied in citrus as described above (Fig. 1, right side). Activation of $ProBs3_{14EBE}$*:GUSi* was highest in combination with *Xcc*306*:avrBs3* (AvrBs3, PthA1, 2, 3 and 4), slightly lower in combination with *Xcc*306 (PthA1, 2, 3, 4) and weakest with *Xcc*306Δ*pthA1–4:avrBs3* (AvrBs3 only). These observations suggest that TALEs act synergistically on $ProBs3_{14EBE}$, which is in agreement with previous findings (Maeder *et al.*, 2013; Perez-Pinera *et al.*, 2013). Notably, the highest GUS levels observed with $ProBs3_{14EBE}$ were more than two-fold higher than those observed with $ProBs3_{1EBE}$, which is possibly a consequence of the tandem-arranged *EBE*s in $ProBs3_{14EBE}$.

Subsequent inoculation of *Xcc*306Δ*pthA1–4:avrBs3* with either $ProBs3_{14EBE}$ or $ProBs3_{1EBE}$ yielded only slightly different levels of GUS activity, suggesting that the insertion of 14 *EBE*s in

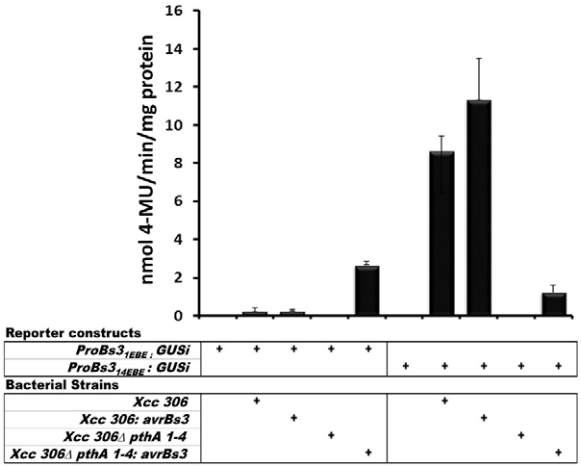

Fig. 1 *Xanthomonas citri* 306 transcription activator-like effector (TALE) recognition by pepper *Bs3* promoter in transient GUSi reporter assay. Grapefruit leaves were infiltrated initially with bacterial suspension [5×10^8 colony-forming units (cfu)/mL] of *Agrobacterium* carrying a GUSi fusion with the *Bs3* native promoter ($ProBs3_{1EBE}$*:GUSi*) or the *Bs3* promoter with 14 effector binding elements (*EBE*s) ($ProBs3_{14EBE}$*:GUSi*), and infiltrated, 5 h later, with *Xcc*306, *Xcc*306Δ*pthA1–4*, *Xcc*306Δ*pthA1–4:avrBs3* or *Xcc*306*:avrBs3*. The infiltrated leaves were assayed for β-glucuronidase (GUS) activity by measuring 4-methyl umbelliferone (4-MU) fluorescence 5 days after subsequent inoculation, expressed as nanomoles 4-MU per minute per milligram protein. Data represent the mean ± standard error (SE) of three independent experiments.

$ProBs3_{14EBE}$ had no strong effect on the functionality of the AvrBs3 binding site. Importantly, the activation of both $ProBs3_{14EBE}$ and $ProBs3_{1EBE}$ was dependent on the presence of matching TALEs, as *Xcc*306Δ*pthA1–4*, which contains no *TALE* genes, did not activate these promoters. In summary, the promoter-reporter assays suggest that the engineered promoter should facilitate the detection of *X. citri* TALEs.

An engineered promoter with *EBE*s matching to multiple known *X. citri* TALEs mediates the recognition of *X. citri* strains with unknown TALE repertoires

We were interested to determine whether $ProBs3_{14EBE}$ could recognize additional *X. citri* strains that possibly contain TALEs that are compatible with *EBE*s of our engineered promoter. To test our assumption, we assayed $ProBs3_{14EBE}$*:GUSi* against a set of 20 *X. citri* strains collected worldwide (Table 1). To do so, the $ProBs3_{14EBE}$*:GUSi* construct was delivered transiently into grapefruit leaves and subsequently inoculated with one of 20 different *X. citri* strains (Fig. 2). We found that all *X. citri* strains, except one (strain 290 from Saudi Arabia), activated $ProBs3_{14EBE}$*:GUSi in planta*, suggesting that the promoter-activating strains have at least one TALE that activates the engineered promoter. Notably, *X. citri* strain 290 fails to produce typical canker symptoms in grapefruit and in all

Table 1 Disease reaction of a range of *Xanthomonas citri* strains* when infiltrated into grapefruit leaves in the presence or absence of engineered *Bs3* promoter constructs *ProBs3*$_{1EBE}$*:avrGf1* and *ProBs3*$_{14EBE}$*:avrGf1*.

		Disease reaction¶		
Strain designation	Origin	No *ProBs3* construct†	*ProBs3*$_{1EBE}$*:avrGf1*‡	*ProBs3*$_{14EBE}$*:avrGf1*§
X. gardneri	Costa Rica	HR**	HR	HR
X. citri-101	Guam	S	HR	HR
X. citri-290	Saudi Arabia	–	–	–
X. citri-46	India	S	HR	HR
X. citri-62	Japan	S	HR	HR
X. citri-106	Australia	S	HR	HR
X. citri-112	China	S	HR	HR
X. citri-131	Maldives Islands	S	HR	HR
X. citri-126	Korea	S	HR	HR
X. citri-257-2	Thailand	S	HR	HR
X. citri-004	Florida, USA	S	HR	HR
X. citri-11#3	Florida, USA	S	HR	HR
X. citri-0018	Florida, USA	S	HR	HR
X. citri-0038	Florida, USA	S	HR	HR
X. citri-98	Florida, USA	S	HR	HR
X. citri-112	Florida, USA	S	HR	HR
X. citri-194	Florida, USA	S	HR	HR
X. citri-2912	Florida, USA	S	HR	HR
X. citri-12815	Florida, USA	S	HR	HR
X. citri-12878	Florida, USA	S	HR	HR

*Pruvost *et al.* (1992); Verniere *et al.* (1998).

†Only *X. citri* strains inoculated onto grapefruit leaves.

‡Grapefruit transiently transformed with *Agrobacterium tumefaciens GV3101* containing *ProBs3*$_{1EBE}$*:avrGf1* and co-infiltrated with *X. citri* strains.

§Grapefruit transiently transformed with *Agrobacterium* containing *ProBs3*$_{14EBE}$*:avrGf1* and infiltrated, 5 h later, with *X. citri* strains. Grapefruit leaves transiently transformed with *A. tumefaciens* alone showed no reaction. Disease reaction was characterized by erumpent lesions appearing after 3 days. HR was determined after 4–5 days.

¶HR, hypersensitive response; S, susceptible; –, no reaction.

**Very weak reaction.

likelihood is unable to grow in grapefruit (Al-Saadi, 2005; Verniere *et al.*, 1998).

AvrGf1 executes cell death in citrus in a TALE-dependent fashion

Thus far, no executor R protein has been identified in citrus and the functionality of known executor R proteins from rice and pepper has not been demonstrated in citrus. Therefore, we tested a microbial Avr protein to activate defences as an alternative to a plant executor R protein. We used the *X. citri* effector AvrGf1, which elicits an HR response in grapefruit (Figueiredo *et al.*, 2011; Rybak *et al.*, 2009) and sweet orange (J. B. Jones *et al.*, unpublished data), to serve as a suitable cell death executor in the specific context of a citrus plant.

The *avrGf1* gene was placed under the transcriptional control of *ProBs3*$_{1EBE}$ in order to examine *Bs3* promoter recognition and cell death in citrus. Young grapefruit leaves were transiently transformed by infiltration with *A. tumefaciens* strains containing this construct (*ProBs3*$_{1EBE}$*:avrGf1*) alone, inoculated with *X. citri* strains individually (*Xcc*306, *Xcc*306Δ*pthA1–4*, *Xcc*306*:avrBs3* or *Xcc*306Δ*pthA1–4:avrBs3*) or treated with combinations of the *ProBs3*$_{1EBE}$*:avrGf1* construct with *X. citri* strains (Fig. 3). Four days after infiltration, leaves were examined for the occurrence of HR. No reaction was apparent in leaf areas infiltrated with the *ProBs3*$_{1EBE}$*:avrGf1* construct only. Infiltration of *ProBs3*$_{1EBE}$*:avrGf1*, followed by inoculation of *Xcc*306, *Xcc*306*:avrBs3* or *Xcc*306Δ*pthA1–4:avrBs3*, produced an HR, whereas inoculation of *Xcc*306Δ*pthA1–4* did not produce an apparent HR. These observations demonstrate a dependence on *Xcc*-mediated delivery of a TALE protein and the presence of a *Bs3* promoter-driven *avrGf1* gene to achieve a hypersensitive defence reaction.

To ensure that the observed HR was indeed triggered by T3SS-delivered effectors, we repeated this experiment using an *X. citri* strain that contains a mutation in the *hrpG* locus (*Xcc306*Δ*hrpG*) and is incapable of delivering effectors by T3SS (Wengelnik *et al.*, 1996). Subsequent inoculation of either *Xcc*306Δ*hrpG* or *Xcc*306Δ*hrpG:avrBs3* with the *Bs3* promoter-driven *avrGf1* gene did not trigger HR in grapefruit leaves 4 days after inoculation (Table 2, Fig. S3, see Supporting Information). These results demonstrate that activation of the *Bs3* promoter by *X. citri* strains requires a functional T3SS.

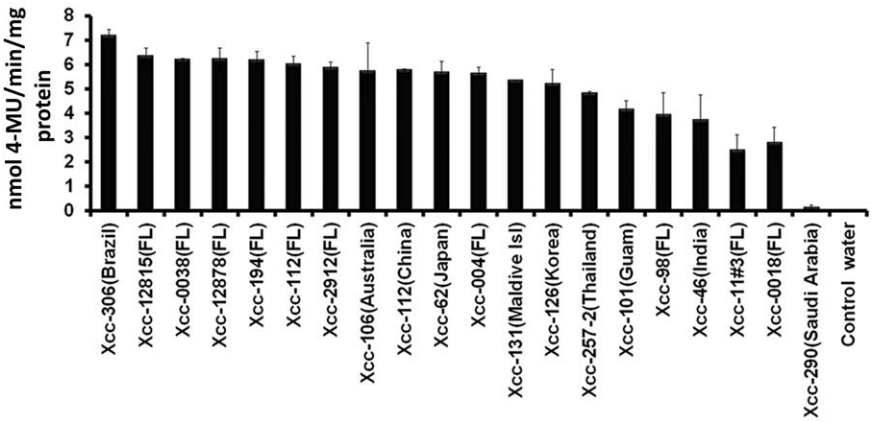

Fig. 2 Induction of *ProBs3*$_{14EBE}$*:GUSi* by a wide range of *Xanthomonas citri* strains. Grapefruit leaves were infiltrated with bacterial suspension [5×10^8 colony-forming units (cfu)/mL] of *Agrobacterium* containing *ProBs3*$_{14EBE}$*:GUSi* in a binary vector, and infiltrated, 5 h later, with bacterial suspensions (adjusted to approximately 5×10^8 cfu/mL) of *Xanthomonas citri* strains or water. The infiltrated leaves were assayed for β-glucuronidase (GUS) by measuring 4-methyl umbelliferone (4-MU) fluorescence 5 days after inoculation, expressed as nanomoles 4-MU per minute per milligram protein. Data represent the mean ± standard error (SE) of three independent experiments.

The results of experiments using the *Bs3* promoter driving either a GUS (Fig. 1) or HR (Fig. 3) reporter both suggest that at least one, but possibly several, of the effectors PthA1–4 are capable of transcriptionally activating the *Bs3* promoter. Yet, these experiments could not distinguish whether the activation of the *Bs3* promoter was caused by the activity of an individual PthA protein or rather by the additive effect of several effectors acting collectively to activate the *Bs3* promoter. To obtain further insights, we therefore transformed the TALE-deficient *Xcc* strain *Xcc*306Δ*pthA1–4* with individual *pthA* genes from *X. citri* strains, and tested which of these PthA-delivering *Xcc* strains triggered HR in combination with *ProBs3*$_{1EBE}$*:avrGf1* (Table 2). We did not observe an HR for any of the individually delivered PthA proteins in combination with *ProBs3*$_{1EBE}$*:avrGf1*, which contains only the AvrBs3 *EBE* (Table 2). This result suggests that the observation of activation of *ProBs3*$_{1EBE}$*:avrGf1* by the intact strain *Xcc*306 is caused by additive effects of PthA1–4 and not by the activity of an individual PthA protein.

To further examine the specificity of the *Bs3* promoter-controlled and AvrGf1-executed HR, we assessed the ability of AvrHah1, a TALE from *X. gardneri* (Schornack *et al.*, 2008), to activate promoter constructs. AvrHah1 also targets the pepper *Bs3* promoter, but with a slightly different DNA sequence specificity than AvrBs3 (Boch *et al.*, 2009). Following infiltration into grapefruit leaves of *Agrobacterium* carrying the *Bs3* promoter-driven '*avrGf1*' gene (*ProBs3*$_{1EBE}$*:avrGf1*), *X. gardneri* strains containing or lacking *avrHah1* (*X. gardneri* Δ*avrHah1*) were subsequently inoculated into the same infiltrated areas. In combination with the *Bs3* promoter-driven *avrGf1* gene, only the *X. gardneri* wild-type, but not the corresponding *avrHah1* mutant, induced an HR (Fig. S4, see Supporting Information).

In summary, analysis of the *Bs3* promoter-driven *avrGf1* gene showed that HR induction by *Xanthomonas* strains is specific and depends on the presence of matching TALEs and a functional T3SS.

An engineered *Bs3* promoter with multiple *EBE*s fused to an executor gene is activated by distinct *X. citri* TALEs

Next, we tested the HR executor *avrGf1* in the context of an engineered *Bs3* promoter, designated as *ProBs3*$_{14EBE}$*:avrGf1*, which contains 14 *EBE*s corresponding to different *X. citri* TALEs. The functionality of this construct was tested *in planta* by subsequent inoculation of *Agrobacterium* containing the engineered promoter in a T-DNA vector and *X. citri* strains that deliver distinct TALE repertoires by the T3SS. Wild-type *Xcc*306 triggered a strong HR in combination with *Agrobacterium* containing the *ProBs3*$_{14EBE}$ construct 4 days after inoculation (Fig. 4A). In contrast, tissue infiltrated with either *Xcc*306 or *ProBs3*$_{14EBE}$*:avrGf1* alone produced typical canker symptoms or no reaction, respectively. Leaf tissue subsequently inoculated with *ProBs3*$_{14EBE}$*:avrGf1* and *Xcc*306Δ*pthA1–4:avrBs3*, but not with *Xcc*306Δ*pthA1–4*, displayed a strong HR, indicating AvrBs3-dependent activation of *ProBs3*$_{14EBE}$*:avrGf1* (Fig. 4B). *Xcc*306Δ*pthA1–4* transconjugants with individual *X. citri TALEs* [*pthA1* (*Xcc*306), *pthA2* (*Xcc*306), *pthA3* (*Xcc*306), *pthA4* (*Xac* 12879), *avrTaw* (*Xau* C340), *pthC* (*Xcc* B69), *pthB* (*Xci* X0053) and pthA^{w} 5.2 (*Xcc* X0053)] also triggered a strong HR in combination with *ProBs3*$_{14EBE}$*:avrGf1*, but not in combination with *ProBs3*$_{1EBE}$*:avrGf1* (Table 2). This observation confirms that only the engineered *Bs3* promoter with the incorporated *EBE*s matching *X. citri* TALEs, but not the wild-type *Bs3* promoter, is capable of mediating the recognition of individual *X. citri* TALE proteins.

Next, we tested a panel of diverse *X. citri* strains with the *ProBs3*$_{14EBE}$*:avrGf1* and *ProBs3*$_{1EBE}$*:avrGf1* constructs. We found that all but the Saudi Arabian *X. citri* strain triggered an HR when subsequently inoculated with *ProBs3*$_{14EBE}$*:avrGf1* and *ProBs3*$_{1EBE}$*:avrGf1* (Table 1). These data demonstrate that our *Bs3* promoter derivative with *EBE*s matching *X. citri* TALEs and also with the native pepper *Bs3* promoter efficiently senses the presence of *X. citri*-delivered TALE proteins.

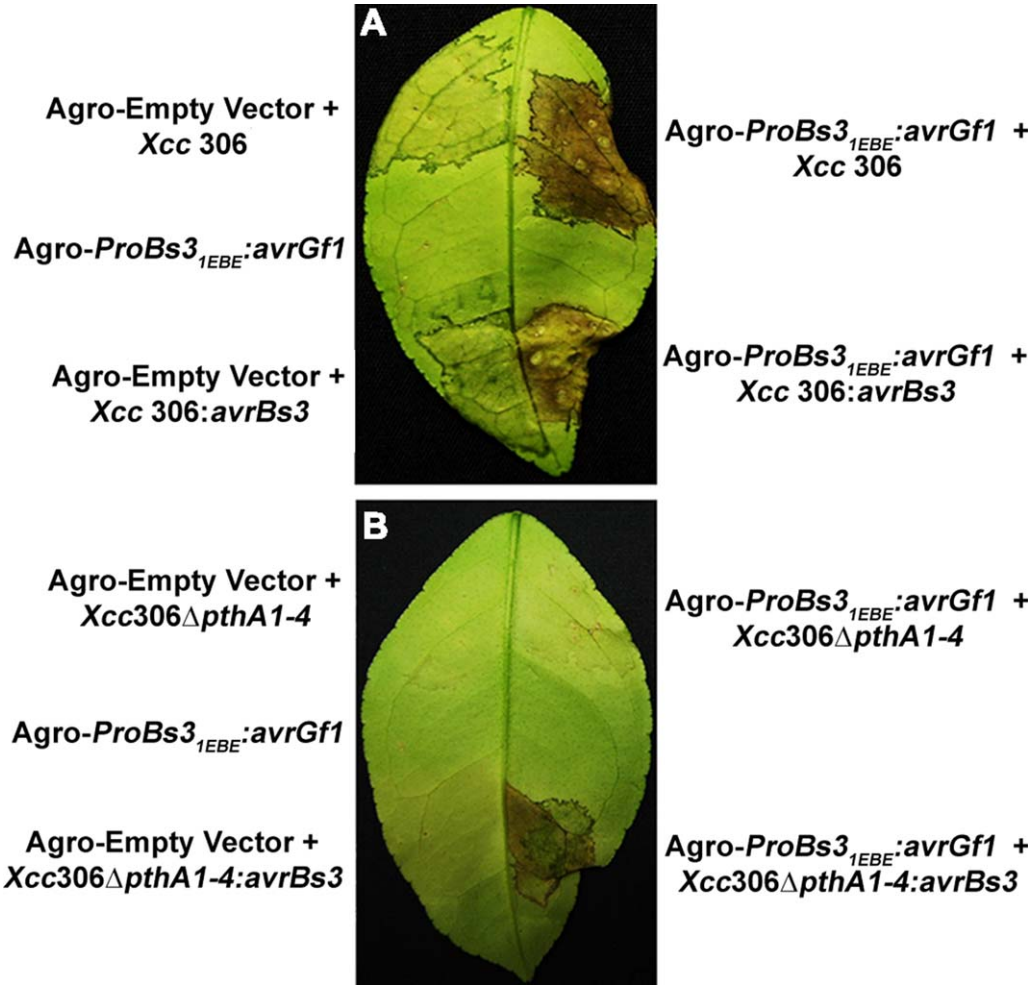

Fig. 3 Expression of the *Bs3* promoter is tightly regulated in grapefruit leaves. Transient assays using the native *Bs3* promoter fused to the *avrGf1* resistance triggering gene (*ProBs3*$_{1EBE}$*:avrGf1*) 3–4 days after inoculation. (A) Grapefruit leaves were infiltrated with a bacterial suspension [5×10^8 colony-forming units (cfu)/mL] of *Agrobacterium* carrying *ProBs3*$_{1EBE}$*:avrGf1* or the empty vector, and infiltrated in the same area, 5 h later, with *Xanthomonas citri* ssp. *citri* 306 containing pLAFR3*::avrBs3* (*Xcc*306*:avrBs3*) or *Xcc*306 containing pLAFR3 empty vector. A hypersensitive response after 3 days was apparent on the right side of the midrib in which *ProBs3*$_{1EBE}$*: avrGf1* was infiltrated prior to infiltration with *Xcc*306 or *Xcc*306 containing *avrBs3*. (B) As a control for *avrBs3* interaction with *ProBs3*$_{1EBE}$*:avrGf1*, grapefruit leaves were infiltrated with *Agrobacterium* carrying *ProBs3*$_{1EBE}$*:avrGf1* or the empty vector, and infiltrated, 5 h later, with *Xcc*306Δ*pthA1–4* containing pLAFR3*::avrBs3* (*Xcc*306Δ*pthA1–4:avrBs3*) or *Xcc*306Δ*pthA1–4* containing pLAFR3 empty vector. A hypersensitive response after 3 days was apparent on the bottom right side of the midrib in which *ProBs3*$_{1EBE}$*: avrGf1* was infiltrated prior to infiltration with *Xcc*306Δ*pthA1–4* containing pLAFR3*::avrBs3*. Representative data of three experiments are shown.

Analysis of the 14 *EBE*s from the engineered *Bs3* promoter uncovers cross-reactivity of *X. citri* TALEs

The *ProBs3*$_{14EBE}$ promoter was constructed with the aim of detecting a broad spectrum of *X. citri* TALEs. Our experiments indeed showed that *ProBs3*$_{14EBE}$ is able to recognize a wide spectrum of *X. citri* TALE proteins (Table 2). We were also interested in examining which of the 14 *EBE*s that were inserted into *ProBs3*$_{14EBE}$ conferred recognition of given *X. citri* TALEs. To functionally dissect these *EBE*s, we constructed 14 distinct *Bs3* promoter derivatives, each containing only one of the 14 *EBE*s present in *ProBs3*$_{14EBE}$ (Fig. 5A). These *Bs3* promoter derivatives were fused upstream of a *GUS* reporter gene. Agrobacteria containing these promoter-reporter T-DNA constructs were infiltrated into *Nicotiana benthamiana* leaves, each in combination with a *35S* promoter-driven *avrBs3*, *pthA1*, *pthA2*, *pthA3* or *pthA4* gene. GUS staining of leaf discs showed that all 14 *Bs3* promoter derivatives produced strong blue staining with the *35S:avrBs3* T-DNA. This promoter activation is expected, as all constructs contain the *Bs3* promoter with its native AvrBs3 binding site. Furthermore, each of the four distinct *35S* promoter-driven *pthA* genes produced strong blue staining with the promoter-reporter construct containing the corresponding *EBE* (Fig. 5B, red circles). Transcriptional activation

Table 2 Pathogen testing of wild-type, mutant and complemented *Xanthomonas citri* ssp. *citri* 306 strains in grapefruit leaves in the presence or absence of *Bs3* engineered constructs *ProBs3*$_{1EBE}$*:avrGf1* and *ProBs3*$_{14EBE}$*:avrGf1*.

		Engineered *Bs3* promoter construct disease reaction[§]		
Strain designation	Origin	No *ProBs3* construct*	*ProBs3*$_{1EBE}$*:avrGf1*[†]	*ProBs3*$_{14EBE}$*:avrGf1*[‡]
X. citri-306	Brazil	S	HR[¶]	HR
X. citri-306:avrBs3	This study	S	HR	HR
*X. citri-306*Δ*pthA1–4*	This study	–	–	–
*X. citri-306*Δ*pthA1–4:avrBs3*	This study	–	HR	HR
*X. citri-306*Δ*hrpG*	This study	–	–	–
*X. citri-306*Δ*hrpG:avrBs3*	This study	–	–	–
*X. citri-306*Δ*pthA1–4:pthA1*	This study	–	–	HR
*X. citri-306*Δ*pthA1–4:pthA2*	This study	–	–	HR
*X. citri-306*Δ*pthA1–4:pthA3*	This study	–	–	HR
*X. citri-306*Δ*pthA1–4:pthA4*	This study	S	–	HR
*X. citri-306*Δ*pthA1–4:avrTaw*	This study	–	–	HR
*X. citri-306*Δ*pthA1–4:pthA*^{w}*5.2*	This study	S	–	HR
*X. citri-306*Δ*pthA1–4:pthC*	This study	–	–	HR
*X. citri-306*Δ*pthA1–4:pthB*	This study	–	–	HR

*Only *X. citri* strains inoculated onto grapefruit leaves.

[†]Grapefruit transiently transformed with *Agrobacterium tumefaciens* GV3101 containing *ProBs3*$_{1EBE}$*:avrGf1* and co-infiltrated with *X. citri* strains.

[‡]Grapefruit transiently transformed with *Agrobacterium* containing *ProBs3*$_{14EBE}$*:avrGf1* and co-infiltrated with *X. citri* strains. Grapefruit leaves transiently transformed with *A. tumefaciens* alone showed no reaction. Disease reaction was characterized by erumpent lesions appearing after 3 days. HR was determined after 4–5 days.

[§]HR, hypersensitive response; S, susceptible; –, no reaction.

[¶]Weak HR.

was expected for promoters containing an *EBE* matching the co-expressed TALE. Yet, in addition to this expected promoter activation, we observed that some TALEs activated promoters that did not contain a corresponding *EBE*, a phenomenon referred to as cross-reactivity. For example, the *X. citri* TALE PthA1 induced strong activation of the promoter containing *EBE*s matching either pB3.7 or PthA*2. PthA4 showed the strongest level of cross-reactivity and activated, like AvrBs3, all promoter-reporter constructs. Sequence alignment of *EBE*s provides a possible explanation for this observation, and suggests that the observed cross-reactivity of TALEs is probably a consequence of the sequence relatedness of the target sites of *X. citri* TALEs. For example, the *EBE* of PthA1, which cross-reacts with promoters containing pB3.7 and PthA*2 *EBE*s, is highly similar to the pB3.7 and PthA*2 *EBE*s, especially in its 5′ region (Fig. 5C). In the case of PthA4, we noted that the 5′ end of its *EBE* is related to the AvrBs3 binding site that is present in the native *Bs3* promoter. This observation rationalizes why PthA4 was capable of activating all *Bs3* promoter-based reporter constructs (Fig. 5B). A similar transient GUS reporter assay in grapefruit leaves corroborated that the *EBE* binding sites in *ProBs3*$_{14EBE}$ were activated by Xcc306 PthA1, 2, 3 and 4. Agrobacteria containing *ProBs3*$_{14EBE}$*:GUSi* promoter-reporter T-DNA constructs were infiltrated into grapefruit leaves, followed by infiltration of Xcc306Δ*pthA*1–4 transconjugants delivering individually PthA1, 2, 3 or 4. Significant activation of *ProBs3*$_{14EBE}$ by all four PthAs was manifested by increased GUS activity, which indicates recognition of Xcc306 PthA1, PthA2, PthA3 and PthA4 by *ProBs3*$_{14EBE}$ (Fig. S5, see Supporting Information).

TALE-induced AvrGf1 expression correlates with reduced growth of the bacterial pathogen

A previous study on plant immunity has revealed that the occurrence of HR does not always correlate with pathogen resistance (Lu *et al.*, 2003). To clarify whether the AvrGf1-induced HR also confers resistance to *X. citri*, we conducted pathogen growth assays. In these assays, grapefruit leaves were infiltrated with either *Agrobacterium* or *Agrobacterium* delivering *ProBs3*$_{1EBE}$*:avrGf1*. Five hours after infiltration of agrobacteria, these leaves were challenge inoculated with either *Xcc*306Δ*pthA1–4* (no TALEs) or *Xcc*306Δ*pthA1–4:avrBs3* (AvrBs3), and *Xcc* growth was quantified at 1, 5, 7 and 12 days after infection. The leaves transiently transformed with *ProBs3*$_{1EBE}$*:avrGf1* and subsequently inoculated with the AvrBs3-delivering strain *Xcc*306Δ*pthA1–4:avrBs3* showed approximately 100-fold less growth relative to the isogenic strain *Xcc*306Δ*pthA1–4*, which lacks AvrBs3, at 12 days after inoculation (Fig. 6A). These data demonstrate that the activation of resistance to *X. citri* is dependent on AvrBs3 and relies on the presence of the *ProBs3*$_{1EBE}$*:avrGf1* construct. We next tested whether *avrGf1* under transcriptional control of the engineered promoter would mediate resistance to *X. citri*

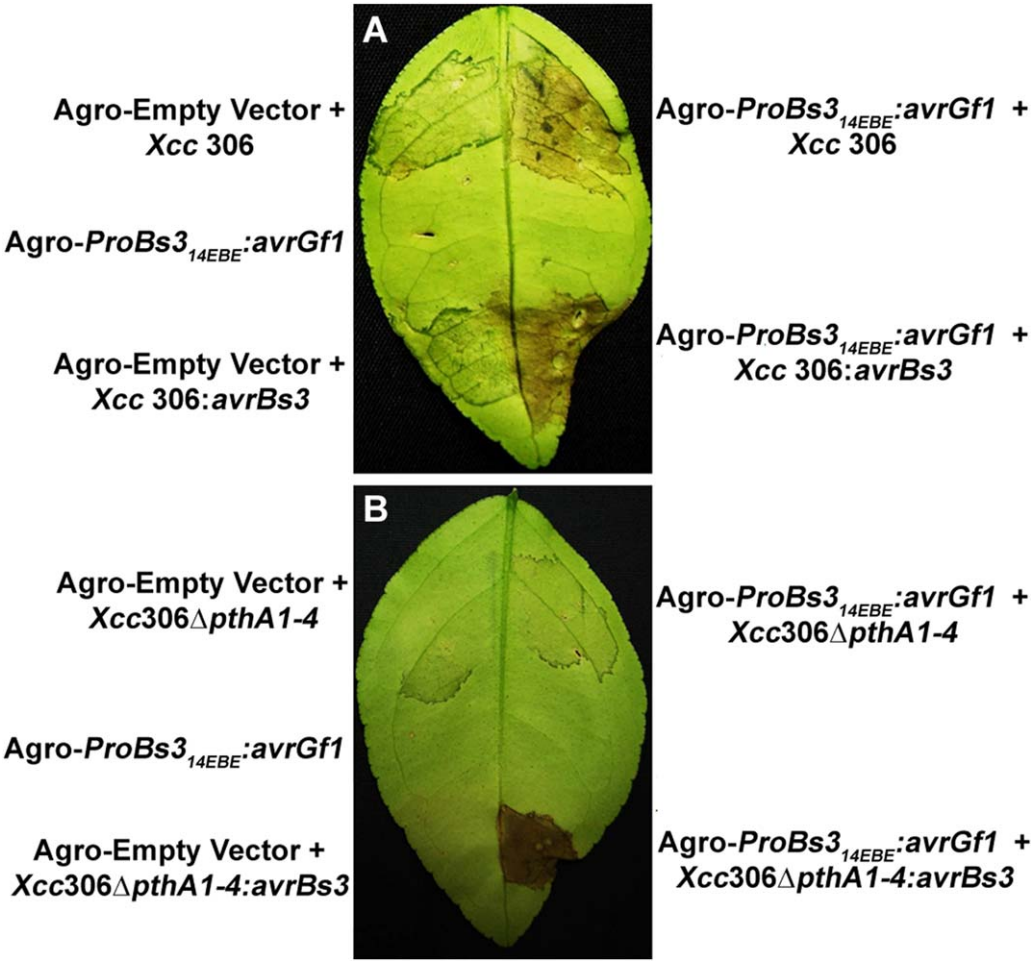

Fig. 4 Expression of *avrGf1* under transcriptional control of an engineered *Bs3* promoter containing 14 effector binding elements (*EBE*s) (*ProBs3*$_{14EBE}$*:avrGf1*) is tightly regulated for resistance in grapefruit leaves. (A) Grapefruit leaves were infiltrated with a bacterial suspension [5×10^8 colony-forming units (cfu)/mL] of *Agrobacterium* carrying *ProBs3*$_{14EBE}$*:avrGf1* or the empty vector, and infiltrated, 5 h later, with *Xanthomonas citri* ssp. *citri* 306 containing pLAFR3*::avrBs3* (*Xcc*306*:avrBs3*) or *Xcc*306 containing pLAFR3 empty vector. A hypersensitive response after 3 days was apparent on the right side of the midrib in which *ProBs3*$_{14EBE}$*:avrGf1* was infiltrated following infiltration with *Xcc*306 or *Xcc*306 containing pLAFR3::*avrBs3*. (B) As a control for an AvrBs3 interaction with *ProBs3*$_{14EBE}$*:avrGf1*, grapefruit leaves were transiently transformed with *Agrobacterium* carrying *ProBs3*$_{14EBE}$*:avrGf1* or the empty vector, and infiltrated, 5 h later, with *Xcc*306Δ*pthA1–4* containing pLAFR3*::avrBs3* (*Xcc*306Δ*pthA1–4:avrBs3*) or *Xcc*306Δ*pthA1–4* containing pLAFR3 empty vector. A hypersensitive response after 3 days was apparent on the bottom right side of the midrib in which *ProBs3*$_{14EBE}$*:avrGf1* was infiltrated prior to infiltration with *Xcc*306Δ*pthA1–4:avrBs3*. Representative data of three experiments are shown.

strains containing PthA4. To do so, grapefruit leaves were infiltrated with either *Agrobacterium* or *Agrobacterium* delivering *ProBs3*$_{14EBE}$*:avrGf1*, followed by challenge inoculation with either *Xcc*306Δ*pthA1–4* (no TALEs) or *Xcc*306Δ*pthA1–4:pthA4* (PthA4). Twelve days after leaves had been transiently transformed with *ProBs3*$_{14EBE}$*:avrGf1* and subsequently inoculated with *Xcc*306Δ*pthA1–4:pthA4*, *X. citri* populations were 100-fold lower relative to those in leaves inoculated with various other combinations of agrobacteria and *X. citri* (Fig. 6B). Similar results were obtained in leaves infiltrated with *ProBs3*$_{14EBE}$*:avrGf1*, followed by challenge inoculation with wild-type *X. citri* (Fig. S6, see Supporting Information). These data demonstrate that activation of an effective defence response relies on PthA4 and the presence of the *ProBs3*$_{14EBE}$*:avrGf1* construct. In a broader context, our studies suggest that, in engineered TALE-inducible *R* genes, microbial Avr proteins can functionally replace executor R proteins.

DISCUSSION

In this study, we generated and functionally validated the use of a microbial avirulence protein under the control of a tightly regulated, engineered promoter construct as a pathogen-inducible executor system, in lieu of an *R* gene, to confer durable and

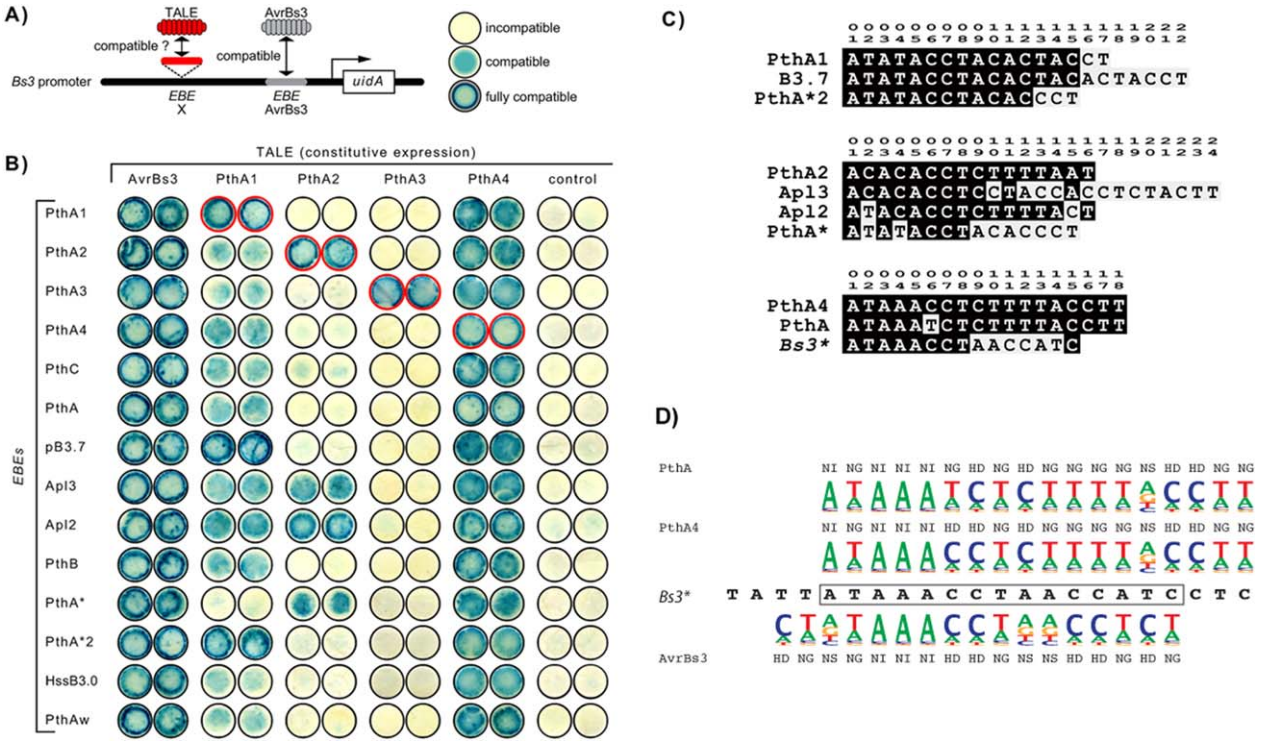

Fig. 5 Functional analysis of 14 individual effector binding elements (*EBE*s) that are present in an engineered *Bs3* promoter derivative using a set of different transcription activator-like effectors (TALEs) from *Xanthomonas citri*. (A) Each of the 14 distinct *EBE*s (*EBE* X) that are tandemly arranged in ProBs3*14EBE* were cloned individually into T-DNA constructs of the *Bs3* wild-type promoter that contains an AvrBs3 binding site (*EBE* AvrBs3). These distinct *Bs3* promoter derivatives were transcriptionally fused to a *uidA* reporter gene. Blue staining of leaves caused by expression of the β-glucuronidase (GUS) reporter provides a proxy for promoter activity. Combination of fully compatible TALE and *EBE* typically results in leaf discs with a dark blue ring with a lighter blue interior (e.g. AvrBs3 with *Bs3* promoter). (B) *35S* promoter-driven T-DNA genes encoding the depicted TALEs (listed horizontally) were delivered together with the depicted promoter-reporter constructs (listed vertically) into *Nicotiana benthamiana* leaves using *Agrobacterium tumefaciens*-mediated transient expression. Delivery of a vector that does not contain a *TALE* gene together with the promoter-reporter construct (control) was used to test whether the observed promoter activities were TALE dependent. At 36 h post-infiltration, the leaves were stained to visualize reporter activity. Leaf discs that are highlighted with a red circle are combinations of PthA1–4 with promoter-reporter constructs that contain the matching *EBE*. (C) Alignment of predicted *EBE*s for TALEs that show cross-reactivity. (D) Alignment of AvrBs3 and its predicted *EBE* in the *Bs3* promoter. RVDs (repeat variable diresidue) of pthA and pthA4 have preferences to *Bs3* promoter *EBE*.

broad-spectrum resistance to the citrus canker pathogen *X. citri*. Conceptually, this synthetic *R* gene is a two-component system with a sensory promoter module controlling expression of the downstream executor module. This two-component concept was proposed as a general means to engineer plants with broad resistance more than two decades ago (De Wit, 1992). Yet, the lack of tightly regulated plant promoters with well-defined *cis*-regulatory elements has been the major limitation of this concept. Engineered TALE-activated *R* genes conceptually fit the envisaged synthetic two-component system by providing fine-tuned promoter-executor modules. Previous studies exploited the nucleotide framework of native TALE-activated pepper and rice *R* genes and inserted additional TALE *EBE*s to expand the repertoire of TALEs that are recognized (Hummel *et al.*, 2012; Römer *et al.*, 2009a; Zeng *et al.*, 2015). Until now, *R* gene engineering approaches have not exploited the modularity of native TALE *R* genes, but have used *R* gene promoters together with their native executor proteins. Because TALE-activated *R* genes have not been identified in citrus, we tested whether the native pepper *Bs3* promoter with the downstream encoded Bs3 protein could be used as a nucleotide framework for integration of *EBE*s matching *X. citri* TALEs. However, *Agrobacterium*-mediated delivery of a *35S* promoter-driven *Bs3* coding sequence did not induce a clear HR phenotype and did not suppress bacterial populations in citrus leaves (data not shown). There could be various technical reasons for the lack of a Bs3-mediated HR in our assay, and there is no conclusive evidence to indicate that Bs3 is incapable of triggering an HR in citrus. However, *Agrobacterium*-mediated delivery of a *35S* promoter-driven *avrGf1* gene is known to trigger HR in citrus (Figueiredo *et al.*, 2011), and presented a possible alternative as a suitable executor module for application in citrus. Indeed, our subsequent studies demonstrated that the expression of *avrGf1* mediates not only an HR, but also reduced growth, of *X. citri* in transient assays (Fig. 6). Although the use of microbial effector

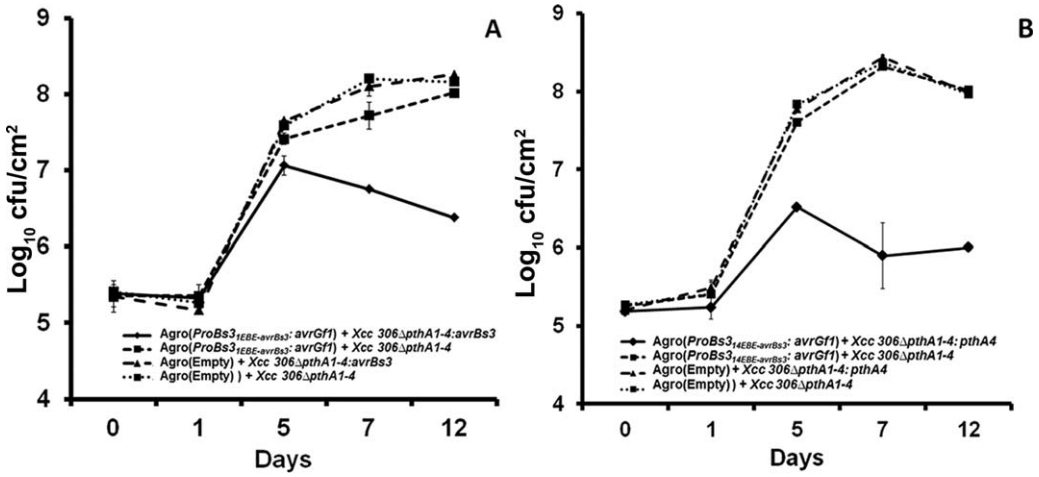

Fig. 6 *In planta* growth of *Xanthomonas citri* 306 (*Xcc*306) on transfection with *ProBs3*$_{1EBE}$*:avrGf1* and *ProBs3*$_{14EBE}$*:avrGf1*. (A) Grapefruit leaves were infiltrated with *Agrobacterium* bacterial suspensions carrying *ProBs3*$_{1EBE}$*:avrGf1* [5×10^8 colony-forming units (cfu)/mL]. Five hours later, the infiltrated areas were infiltrated with bacterial suspensions (5×10^7 cfu/mL) of *Xcc306ΔpthA1–4:avrBs3*. Expression of AvrGf1 by AvrBs3 activation on *ProBs3*$_{1EBE}$ leads to a hypersensitive reaction, reducing the growth of *Xcc*306 at 5 days after infection onwards. Experimental controls were *Agrobacterium* carrying empty vector with *Xcc*306Δ*pthA1–4:avrBs3*; *Agrobacterium* with and without *ProBs3*$_{1EBE}$*:avrGf1* infiltrated with *Xcc*306Δ*pthA1–4* did not reduce the population. (B) Similarly, grapefruit leaves were infiltrated with an *Agrobacterium* bacterial suspension carrying *ProBs3*$_{14EBE}$*:avrGf1* (5×10^8 cfu/mL). Five hours later, the infiltrated areas were infiltrated with bacterial suspensions (5×10^7 cfu/mL) of *Xcc*306Δ*pthA1–4:pthA4*. The expression of AvrGf1 by PthA4 activation on *ProBs3*$_{14EBE}$ leads to a hypersensitive reaction, reducing the growth of *Xcc*306 at 5 days after infection onwards. Experimental controls were *Agrobacterium* carrying empty vector with *Xcc*306Δ*pthA1–4:pthA4*; *Agrobacterium* with and without *ProBs3*$_{1EBE}$*:avrGf1* infiltrated with *Xcc*306Δ*pthA1–4* did not reduce the population. Data represent the mean ± standard error (SE) of three experimental replicates.

genes as executor modules has been suggested previously (Boch *et al.*, 2014), to the best of our knowledge, our studies represent the first experimental demonstration of the functional linking of a TALE-inducible *R* gene promoter to an HR-inducing microbial effector protein to confer potential pathogen resistance in a crop plant. Given that a large number of microbial effector genes have been cloned, each activating a pathogen-specific defence response in a given host plant, the use of microbial effector genes as executor modules will substantially broaden the range of pathogens and host plants for which a synthetic *R* gene can be engineered.

For maximum durability, engineered *R* gene promoters with multiple TALE *EBE*s should be designed to recognize a spectrum of TALEs that are present in a given pathogen population. Yet, the implementation of *EBE*s is limited to TALEs for which sequence information is available. Analysis of our *EBE*-enriched promoter showed that 19 of 20 *X. citri* strains transcriptionally activated this promoter. In this assay, *X. citri* strain 290 was the exception and did not activate the promoter (Fig. 2). This observation is in agreement with previous studies showing that *X. citri* strain 290 from Saudi Arabia fails to produce typical canker symptoms in grapefruit and, in all likelihood, is unable to grow in grapefruit (Al-Saadi, 2005; Verniere *et al.*, 1998). The finding that *X. citri* strain 290 also did not produce any canker lesions (Table 1) possibly suggests that this *X. citri* strain lacks functional TALE proteins or a functional T3SS. This is somewhat reminiscent of weakly pathogenic strains of the rice pathogen *Xanthomonas oryzae* which also lack TALE proteins (Triplett *et al.*, 2011).

Our observation that the engineered promoter with tandem arranged *EBE*s recognizes most, but not all, strains of the pathogen population is again similar to previous studies in rice, in which integration of multiple tandem-arranged TALE *EBE*s into the rice *Xa27* and *Xa10* promoters also facilitated the recognition of many, but not all, strains tested (Hummel *et al.*, 2012; Zeng *et al.*, 2015). Therefore, it is important to design a synthetic promoter in citrus based on TALE repertoires to ensure broad recognition and to avoid putting pressure on individual TALEs to evolve. Given that TALEs are generally crucial to the pathogenicity of *X. citri* strains, TALE-lacking strains might escape detection by our engineered promoter, but do not pose a threat because of their weak pathogenicity (Hu *et al.*, 2014; Rybak *et al.*, 2009).

The *ProBs3*$_{14EBE}$*:avrGf1* construct was shown to confer broad resistance to a large number of *X. citri* strains from Florida and other geographical locations (Pruvost *et al.*, 1992; Verniere *et al.*, 1998). Although these *X. citri* TALEs have not been characterized, they elicit an HR when subsequently inoculated into grapefruit leaves with *ProBs3*$_{14EBE}$*:avrGf1*. Subsequent inoculations of *ProBs3*$_{14EBE}$*:avrGf1* together with these diverse *X. citri* strains demonstrated induction of a complex promoter resulting in HR by a broad range of *X. citri* strains (Table 1). The notable exception was *X. citri* strain 290, which, as discussed above, is weakly pathogenic.

We and others have added individual TALE *EBE*s to promoters to increase the recognition spectrum of an engineered *R* gene. Another strategy for the design of a synthetic *R* gene targeting TALEs would be to utilize the whole promoter region of a given *S* gene rather than individual *EBE*s only. We utilized this approach for the citrus canker bacterium that targets the citrus *LOB1* (*Lateral organ boundaries 1*) gene as the major *S* gene (Hu *et al.*, 2014). Six *X. citri* PthA4 homologues were identified with identical or overlapping *EBE*s in the *LOB1* promoter. A promoter-reporter gene containing the *LOB1* promoter segment was transcriptionally activated by all six *X. citri* PthAs. Similarly, a fusion of the *LOB1* promoter segment to an *avrGf1* homologue triggered HR in grapefruit leaves on subsequent inoculation with *X. citri* (J. B. Jones *et al.*, unpublished data). One risk associated with this approach is that the housekeeping functions of such genes may mean that *cis*-regulatory elements in the *S* promoter may be found in these promoter segments, causing undesired expression, i.e. leakiness.

We have not currently achieved the production of stable transgenics with $ProBs3_{1EBE}$*:avrGf1* or $ProBs3_{14EBE}$*:avrGf1* in grapefruit or sweet orange. Transformation of citrus species is difficult and generally suffers from low efficiency, impeding the recovery of the desired transformants in which the transgene is inserted into a suitable genomic region that permits TALE-inducible transgene expression. In addition, it is possible that spurious expression of the microbial effector during transformation or differentiation impedes the recovery of positive transformants. This possibility is supported by the observation that stable transgenic lines were obtained with $ProBs3_{14EBE}$*:avrGf1* in Carrizo citrange, a citrus rootstock with higher efficiency of transformation and which does not demonstrate an HR in response to AvrGf1 (Shantharaj *et al.*, unpublished data). The results obtained by Hummel *et al.* (2012) with rice demonstrate that it is possible to successfully engineer resistance by adding EBEs to an executor gene promoter, but that it may be necessary to optimize promoter sequences to avoid activation by other factors. There is a good indication that addressing this technical issue is feasible and can lead to the generation of stably transformed commercial citrus lines that can produce broad resistance to this damaging disease, for which genetic solutions are lacking.

TALEs and their interaction with DNA have been studied not only in the context of plant–microbe interactions, but also in the context of applied biotechnology (Zeng *et al.*, 2015). As a result of the concerted efforts of numerous research groups, the molecular mechanisms of TALE–DNA interaction are fairly well elucidated. Indeed, integration of individual predicted *EBE*s into a promoter is a routine procedure that yields TALE-inducible genes with high success rates (Hummel *et al.*, 2012). Yet, we noted that the functional consequences resulting from the combination of multiple TALEs or multiple *EBE*s are still difficult to predict. For example, the *X. citri* strain *Xcc*306Δ*pthA1–4:avrBs3*, which delivers AvrBs3, activates $ProBs3_{1EBE}$, whereas the isogenic variant *Xcc*306*:avrBs3*, which delivers AvrBs3 and *X. citri* TALEs PthA1–4, activates $ProBs3_{1EBE}$ to a much lesser extent (Fig. 1). This observation is somewhat unexpected and suggests that PthA1–4 proteins somehow interfere with AvrBs3-mediated promoter activation. $ProBs3_{14EBE}$, the *EBE*-enriched derivative of $ProBs3_{1EBE}$, contains not only an AvrBs3 binding site, but also distinct *EBE*s for PthA1–4, and was most strongly activated by *Xcc*306*:avrBs3* and to a slightly lesser extent by *Xcc*306Δ*pthA1–4:avrBs3*. Thus, PthA1–4 have an antagonistic effect on promoter activation in the context of $ProBs3_{1EBE}$, but act synergistically in the context of $ProBs3_{14EBE}$. Although, in most cases, our observations can be rationalized, it also becomes clear that further studies are needed to clarify the molecular basis of the observed synergistic and antagonistic effects on promoter activation. In the long run, such studies will promote a more rationalized assembly of *EBE*s in a given promoter.

EXPERIMENTAL PROCEDURES

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table S2 (see Supporting Information). *Agrobacterium* binary plasmid constructs were generated through standard cloning methods as described previously (Römer *et al.*, 2009a). Deletion mutants were created as described previously (Hu *et al.*, 2014). Transconjugants in *X. citri* strains were prepared by triparental mating as described previously (De Feyter *et al.*, 1990).

Plant material and plant inoculations

Grapefruit cv. Duncan (*Citrus paradisi*) plants were grown in the glasshouse at temperatures ranging from 20 to 35 °C, 12-h day/12-h night photoperiod and relative humidity (RH) of 60%. Young leaves (2–3-week-old leaves after pruning) were inoculated with a hypodermic needle and syringe into the abaxial surface of the leaf. For the preparation of bacterial suspensions of *X. citri* strains, 18-h cultures were harvested from solid medium, suspended in sterile tap water and standardized to an optical density at 600 nm (OD_{600}) of 0.3, corresponding to 5×10^8 colony-forming units (cfu/mL).

In planta Agrobacterium-mediated transient assays on citrus

GUS activity, HR and cell death induction were tested in engineered binary constructs with one *EBE* ($ProBs3_{1EBE}$*:GUSi* or *avrGf1*) or 14 *EBE*s ($ProBs3_{14EBE}$*:GUSi* or *avrGf1*) carried in *Agrobacterium* on intact grapefruit leaves. Briefly, *A. tumefaciens* strains harbouring the desired constructs were infiltrated at $OD_{600} = 0.3$, and the same infiltrated areas were subsequently inoculated, 5 h later, with *X. citri* suspensions at 5×10^8 cfu/mL. The plants were maintained in the growth room with a constant day/night temperature of 28 °C, 12-h day/12-h night photoperiod and RH of 60%, and examined for cell death symptoms, bacterial population dynamics in leaf tissue and/or GUS activity.

Quantification of GUS activity

GUS activity was measured using the fluorescent substrate 4-methylumbelliferyl β-D-glucuronide (MUG, Sigma, Saint Louis, Missouri, USA) according to the standard protocol of Jefferson *et al.* (1987) with some modifications. Two leaf discs (0.5 cm in diameter) were collected using a cork borer, and homogenized with a mortar and pestle in 500 μL of GUS extraction buffer containing MUG. Extracted samples were centrifuged at 12000 × *g* for 5 min at 4 °C, and the supernatant was incubated at 37 °C. One hour after incubation, an aliquot of 25 μL was transferred and the reaction was stopped with 225 μL of 0.2 M sodium carbonate. The fluorescence of 4-methyl umbelliferone (4-MU) released from each sample reaction was measured (excitation, 365 nm; emission, 455 nm) using a CytoFluor II fluorescence multiwell plate reader (Perspective Biosystems, Framingham, MA, USA). Fluorescence units were quantified against the 4-MU standard curve. The protein concentration in each sample was determined using Biorad protein reagent (Hercules, CA, USA). GUS activity was expressed as nanomoles of 4-MU per minute per milligram protein.

Analysis of cross-reactivity of TALEs in *N. benthamiana*

Nicotiana benthamiana plants were grown in a glasshouse at 60%–70% humidity at 22 °C during the day (16 h of light) and 18 °C at night; 6–8-week-old plants were used for inoculation. For GUS assays, *A. tumefaciens* strains were grown overnight in YEB (0.5% beef extract; 0.1% yeast extract; 0.5% peptone; 0.5% sucrose; 2mM MgSO4) medium, collected by centrifugation, resuspended in water and adjusted to $OD_{600} = 0.8$. *Agrobacterium tumefaciens* strains containing *35S* promoter-driven *TALE* genes and *Bs3* promoter derivatives fused to the reporter gene *uidA* (GUS) were mixed in a 1 : 1 ratio before inoculation. After 36 h, leaf discs were harvested, infiltrated (vacuum infiltration) with GUS-staining solution [100 mM sodium phosphate (pH 7), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide] and incubated at 37 °C overnight. Leaf discs were cleared in ethanol.

Measurement of *X. citri* populations in transiently transformed grapefruit

For the measurement of *Xcc*306 growth *in planta*, intact grapefruit leaves were initially inoculated with *Agrobacterium* carrying one of the engineered resistance constructs and subsequently inoculated with *Xcc*306 as described above. At 0, 1, 5, 7 and 12 days after infiltration, bacterial populations were measured in each of three leaves. An infiltrated leaf disc (0.5 cm in diameter) was placed in 1 mL of sterile tap water and triturated. Ten-fold dilutions with sterile tap water were made and 50 μL were plated on nutrient agar. Bacterial colonies were counted and populations were calculated. Experiments were repeated three times.

Data analysis

All of the data were averaged from three separate experiments unless mentioned otherwise and further analysed by one-way analysis of variance (ANOVA) using Microsoft Excel 2010® and statistical package JMP®10. The data means were considered to be significantly different at the probability $P \leq 0.05$.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 (A) *ProBs3*$_{1EBE}$ sequence, *Bs3* promoter with transcription activator-like effector (TALE) binding site (*EBE*$_{AvrBs3}$) is in bold and underlined. (B) Representation of *ProBs3*$_{1EBE}$ fusion with GUSi (uidA gene containing an intron). The native pepper *Bs3* promoter, having effector binding element (*EBE*) (*ProBs3*$_{1EBE}$) for the *Xanthomonas euvesicatoria* TALE *AvrBs3*, is represented by a blue arrow. or (C) *ProBs3*$_{1EBE}$ fusion with *avrGf1* (*avrGf1* gene accession DQ275469.1).

Fig. S2 (A) *ProBs3*$_{14EBE}$ sequence. A synthetic sequence (lower case letters) introduced into the pepper *Bs3* promoter between two restriction sites (underlined). The 14 effector binding elements (*EBEs*) matching to given transcription activator-like effectors (*TALEs*) are displayed in coloured capital letters with the corresponding TALE name above. (B) Representation of *ProBs3*$_{14EBE}$ fusion with GUSi (uidA gene containing an intron), *EBEs* for citrus TALE are shown in blue and the pepper *EBE*$_{AvrBs3}$ binding site is shown in green. or (C) *ProBs3*$_{14EBE}$ fusion with avrGf1 (*avrGf1* gene accession DQ275469.1).

Fig. S3 Analysis of *Bs3* promoter induction using *Xanthomonas citri* strains and corresponding *hrpG* mutants. Grapefruit leaves infiltrated with a bacterial suspension [5×10^8 colony-forming units (cfu)/mL] of *Agrobacterium* containing *ProBs3*$_{1EBE}$*:avrGf1* (*avrGf1*) or empty vector, and infiltrated, 5 h later, with bacterial suspensions (5×10^8 cfu/mL) of *Xcc*306Δ*hrpG* or *Xcc*306Δ*hrpG*:*avrBs3*. Bacterial suspensions were infiltrated into designated areas in the leaf: *Xcc*306Δ*hrpG* (A, top left); *ProBs3*$_{1EBE}$*:avrGf1* + *Xcc*306Δ*hrpG* (A, top right and bottom right); *ProBs3*$_{1EBE}$*:avrGf1* (A and B,

bottom left); *Xcc*306Δ*hrpG:avrBs3* (B, top left); *ProBs3*$_{1EBE}$*:avrGf1* + *Xcc*306Δ*hrpG:avrBs3* (B, right). No hypersensitive responses were observed. Representative images of three independent experiments.

Fig. S4 Analysis of *Bs3* promoter activation using AvrHah1-delivering *Xanthomonas citri* strains. Grapefruit leaves were inoculated by infiltration with suspensions [5×10^8 colony-forming units (cfu)/mL] of *Agrobacterium* containing binary construct *ProBs3*$_{1EBE}$*:avrGf1* or empty vector, and infiltrated, 5 h later, with *X. gardneri* (*avrHah1*) or *X. gardneri* (Δ*avrHah1*). A strong hypersensitive response (HR) was observed 4 days after inoculation only in the area in which *X. gardneri* (*avrHah1*) was infiltrated 5 h after *ProBs3*$_{1EBE}$*:avrGf1*. Representative images of three independent experiments.

Fig. S5 *Xanthomonas citri* ssp. *citri* 306 transcription activator-like effector (TALE) (PthA1, PthA2, PthA3, PthA4) recognition efficiency to the predicted effector binding elements in engineered reporter construct *ProBs3*$_{14EBE}$*:GUSi*. Grapefruit leaves were infiltrated with a bacterial suspension [5×10^8 colony-forming units (cfu)/mL] of *Agrobacterium ProBs3*$_{14EBE}$*:GUSi* construct, and infiltrated, 5 h later, with *Xcc*306Δ*pthA1–4* transconjugants individually containing pLAFR3 or pLAFR3 with *pthA1*, *pthA2*, *pthA3* or *pthA4* to understand individual TALE interactions mediating promoter recognition. The infiltrated leaves were assayed for β-glucuronidase (GUS) expression by measuring 4-methyl umbelliferone (4-MU) fluorescence after 5 days, expressed as nanomoles 4-MU per minute per milligram protein. Data represent the mean ± standard error (SE) of three independent experiments.

Fig. S6 *In planta* growth of *Xcc*306 on transfection with *ProBs3*$_{14EBE}$*:avrGf1*. Grapefruit leaves were infiltrated separately with *Agrobacterium* bacterial suspension carrying *ProBs3*$_{14EBE}$*:avrGf1* and empty vector at 5×10^8 colony-forming units (cfu)/mL. Five hours later, the infiltrated areas were infiltrated with bacterial suspensions (5×10^8 cfu/mL) of *Xcc*306. At 0, 2, 4 and 6 days after infiltration (dai), *Xcc*306 bacterial populations were quantified in each of three leaves. An infiltrated leaf disc (0.5 cm in diameter) was placed in 1 mL of sterile tap water and triturated. Expression of AvrGf1 by PthAs of *Xcc*306 caused a reduction in growth of *Xcc*306 at 2 dai onwards. Experimental control: *Agrobacterium* carrying empty vector infiltrated with *Xcc*306 did not reduce the population. Data represent the mean ± standard error (SE) of three experimental replicates.

Table S1 Known transcription activator-like effectors (TALEs) in citrus canker strains and corresponding effector binding elements (*EBE*s) present in the 14-EBE engineered Bs3 promoter constructs used in this study.

Table S2 Bacterial strains and plasmids used in this study.